

Activation of calcineurin underlies altered trafficking of $\alpha 2$ subunit containing GABA_A receptors during prolonged epileptiform activity



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ABSTRACT

Fast inhibitory signalling in the mammalian brain is mediated by gamma-aminobutyric acid type A receptors (GABA_ARs), which are targets for anti-epileptic therapy such as benzodiazepines. GABA_ARs undergo tightly regulated trafficking processes that are essential for maintenance and physiological modulation of inhibitory strength. The trafficking of GABA_ARs to and from the membrane is altered during prolonged seizures such as in Status Epilepticus (SE) and has been suggested to contribute to benzodiazepine pharmacoresistance in patients with SE. However, the intracellular signalling mechanisms that cause this modification in GABA_AR trafficking remain poorly understood. In this study, we investigate the surface stability of GABA_ARs during SE utilising the low Mg²⁺ model in hippocampal rat neurons. Live-cell imaging of super ecliptic pHluorin (SEP)-tagged $\alpha 2$ subunit containing GABA_ARs during low Mg²⁺ conditions reveals that the somatic surface receptor pool undergoes down-regulation dependent on N-methyl-D-aspartate receptor (NMDAR) activity. Analysis of the intracellular Ca²⁺ signal during low Mg²⁺ using the Ca²⁺-indicator Fluo4 shows that this reduction of surface GABA_ARs correlates well with the timeline of intracellular Ca²⁺ changes. Furthermore, we show that the activation of the phosphatase calcineurin was required for the decrease in surface GABA_ARs in neurons undergoing epileptiform activity. These results indicate that somatic modulation of GABA_AR trafficking during epileptiform activity *in vitro* is mediated by calcineurin activation which is linked to changes in intracellular Ca²⁺ concentrations. These mechanisms could account for benzodiazepine pharmacoresistance and the maintenance of recurrent seizure activity, and reveal potential novel targets for the treatment of SE.

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1. Introduction

GABA_A Receptors (GABA_ARs) are ligand-gated chloride permeable ion channels which mediate both phasic (synaptic) and tonic (extrasynaptic) inhibitory neurotransmission in the central nervous system (Jacob et al., 2008; Luscher et al., 2011). They assemble from five subunits, the composition of which determines the receptors functional and pharmacological properties and the specific location on the neuronal membrane (Luscher et al., 2011; Jacob et al., 2008). GABA_ARs containing the $\gamma 2$ subunit mediate synaptic transmission (in contrast to extrasynaptic receptors located away from the synapse) and are a target for benzodiazepines (Pritchett et al., 1989).

The enrichment of GABA_ARs in subcellular compartments such as the axon initial segment (AIS) has been reported for the $\alpha 2$ subunit and although both $\alpha 1$ and $\alpha 2$ subunits are found at the synapse in dendrites, a minority of GABA_ARs in the AIS contain the $\alpha 1$ subunit (Panzanelli et al., 2011; Brünig et al., 2002). GABA_ARs undergo dynamic movement within the cellular membrane. Lateral diffusion facilitates trafficking and assures the appropriate surface localisation of the receptor (Mukherjee et al., 2011), while trafficking to and from the membrane through exocytotic and endocytotic processes allows constant maintenance of the inhibitory synaptic receptor pool (Bogdanov et al., 2006; Kittler et al., 2000, 2004). Altered neuronal activity causes surface GABA_ARs to undergo plasticity-induced trafficking changes. These are mediated by alterations in the activity of protein phosphatases and kinases which are linked to changes in intracellular Ca²⁺ (Muir et al., 2010; Bannai et al., 2009; Saliba et al., 2012; Luscher et al., 2011; Jacob et al., 2008; Petrini et al., 2014).

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SE evolves rapidly and dynamically, manifesting as a prolonged and self-sustaining seizure with significant morbidity and mortality (Lothman, 1990; Dodrill and Wilensky, 1990; Sutter et al., 2013). This distinct condition can occur in patients with previous epilepsy or may occur *de novo* as a result of acute neurological disorders (Trinka et al., 2012). As SE evolves, the patient's response to treatment with benzodiazepines decreases progressively which rapidly results in benzodiazepine pharmacoresistance. This may lead to refractory SE, a pathological state in which seizures are not sopped by first- or second-line anticonvulsant therapies.

To unravel the role of benzodiazepine pharmacoresistance associated with SE patients, studies have addressed whether the trafficking of GABA_ARs to and from the cellular membrane is altered during models of SE (Naylor et al., 2005; Goodkin et al., 2005; Blair et al., 2004). Interestingly, it has been suggested that GABA_ARs are subjected to subunit-specific trafficking during prolonged depolarisation. GABA_ARs containing the synaptic subunits $\beta 2/3$ and $\gamma 2$ undergo internalisation whereas those containing the extra-synaptic δ subunit remain unchanged (Goodkin et al., 2008). Despite recent studies, the temporal dynamics of GABA_AR trafficking have not been investigated using live-cell imaging. Moreover, whether endocytosis occurs preferentially in distinct compartments such as dendrites or soma remains unclear. It is not known which molecular pathways underlie this subunit-specific trafficking of GABA_ARs. Furthermore, it remains to be determined whether Ca^{2+} and its intracellular signalling cascades play a significant role in the modulation of GABAergic inhibition during SE.

To address the molecular mechanisms underlying altered GABA_AR trafficking during SE, we used a live-cell imaging approach to examine the surface stability of GABA_ARs in hippocampal neurons. We induced prolonged epileptiform bursting activity *in vitro* by exposing neurons to artificial cerebrospinal fluid (aCSF) lacking Mg^{2+} (Mangan and Kapur, 2004; Sombati and Delorenzo, 1995). Using this model, we show a decrease in somatic surface GABA_ARs that is dependent on NMDAR activity and the Ca^{2+} -dependent phosphatase, calcineurin. Furthermore, we show that epileptiform activity alters intracellular Ca^{2+} concentrations, which correlates with the decrease of GABA_ARs from the surface possibly contributing to pathological signalling during SE.

2. Materials and methods

2.1. Constructs

The N-terminally tagged GABA_A $\alpha 2\text{-SEP}$ DNA was a kind gift from S. Moss (Tufts University, Cambridge, MA) and has been described previously (Tretter et al., 2008).

2.2. Cell culture and transfection

All animal experiments were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986. All efforts were made to minimise animal suffering and to reduce the number of animals used. Dissected hippocampi of P0 rat pups or E18 embryos were immediately placed in ice-cold dissection buffer (HBSS (Invitrogen)) and washed once. Using trypsin (0.25%) tissue was digested for 10 min before trituration in ~2 ml of attachment medium. Neurons were plated onto poly-L-lysine (Sigma) coated coverslips (500 $\mu\text{g}/\text{ml}$). For nucleofection, hippocampal neurons were nucleofected with GABA_A $\alpha 2\text{SEP}$ plasmid DNA. Neurons were centrifuged and the cell pellet was resuspended in 100 μl transfection buffer (135 mM KCl, 10 mM HEPES-pH 7.3, 2 mM MgCl_2 , 5 mM EGTA, 0.2 mM CaCl_2) and transfected using a single cuvette AMAXA system (Lonza, programme O-003 or AK-009). Neurons were left to develop at 37 °C and 95% O_2 , 5% CO_2 in maintenance medium [Neurobasal (Invitrogen), B27 Supplement (Invitrogen), 0.6% Glucose (Sigma), 2 mM Glutamine (Invitrogen) and Penicillin–Streptomycin] for 14–21 DIV before imaging.

2.3. Live-cell imaging

Live-cell imaging was performed on an upright Olympus microscope (BX51WI) coupled to an EM-CCD camera (Ixon, Andor). Cells were imaged with a water-immersion 60 \times objective (Olympus). Excitation was provided by an X-cite 120Q light source (Lumen Dynamics). Appropriate filters were used (in nm): Excitation: 470/40; Emission: 525/50; Dichroic: 495, long pass. The image pixel scale was calculated by dividing the camera pixel size (16 μm) by the lens magnification (60 \times)

yielding a pixel size of 0.27 μm . Before constant perfusion with a Cole–Parmer Master-Flex pump (~4 ml/min), aCSF (126 mM NaCl, 24 mM NaHCO_3 , 10 mM D-Glucose, 2.5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 1 mM NaH_2PO_4 , 5 mM Sodium Pyruvate) was pre-equilibrated for 20 min with 95% O_2 and 5% CO_2 to establish a pH of 7.4. Temperature of the waterbath was constantly measured using a digital Thermometer (Hanna Instruments) and maintained at 37 °C. Any focus drift was corrected manually. Protocols were adapted to achieve minimal bleaching conditions. Imaging of SEP-tagged GABA_ARs was done for 60 min at a rate of one frame every 20 s (180 frames, 48.8 ms exposure, no averaging). For imaging of intracellular Ca^{2+} using fluo4 (1 μM , Molecular Probes, Invitrogen) hippocampal neurons were incubated for 30 min at 37 °C. After washing twice, fluo4-imaging was done for 60 min (720 frames, 5 ms exposure, no averaging) at 1 frame every 5 s.

2.4. Cell-attached recording

Cell-attached recordings were made on transfected hippocampal neurons at 13 DIV using an Axopatch 200B amplifier (Molecular Devices) and pClamp software. Cells were visualised using an upright Olympus BX50WI microscope equipped with a 40 \times water-immersion objective and infrared optics. Recording electrodes were pulled from standard-walled borosilicate glass capillaries (Warner Instruments) and filled with aCSF. Gigaseal cell attached recordings were made in voltage-clamp mode at –70 mV; the cells were constantly perfused with aCSF. To block currents during recording NBQX disodium salt (20 μM , Abcam) and dAPV (D-(–)-2-Amino-5-phosphonopentanoic acid, 25 μM , TOCRIS) were added to the perfusion solution.

2.5. Low Mg^{2+} and drug treatments

To induce epileptiform bursting activity, aCSF without Mg^{2+} but 2 μM glycine (126 mM NaCl, 24 mM NaHCO_3 , 10 mM D-Glucose, 2.5 mM KCl, 2 mM CaCl_2 , 1 mM NaH_2PO_4 , 5 mM sodium pyruvate, 2 μM glycine) was used (Blair et al., 2004). We confirmed previous studies (Robinson et al., 1993; Mangan and Kapur, 2004; Delorenzo et al., 1998) that low Mg^{2+} results in cellular burst spiking that is dependent upon glutamateric transmission (Sup. Fig. 2). Moreover, action potentials were associated with post-synaptic currents, indicating that the bursting was the result of network activity (Sup. Fig. 2). The mitochondrial substrate sodium pyruvate was supplemented to reduce neuronal death (Kovac et al., 2012). Transfected hippocampal neurons were perfused with control aCSF for 3.3 min (10 frames, baseline), followed by either low Mg^{2+} treatment or continued perfusion with aCSF with Mg^{2+} (control) for 60 min. To block NMDAR activity during low Mg^{2+} treatment, the NMDAR blocker dAPV (25 μM , TOCRIS) was used continuously throughout the low Mg^{2+} treatment without preincubation. For low Mg^{2+} /NMDA treatment, NMDA (30 μM , TOCRIS) was added to the low Mg^{2+} medium and applied continuously for 60 min. To block the activity of the Ca^{2+} dependent phosphatase calcineurin, cells were pre-incubated with calcineurin autoinhibitory peptide (Terada et al., 2003) (50 μM , Calbiochem) for 25 min at 37 °C and imaged (without application of the peptide during perfusion) either during control or low Mg^{2+} treatment (Muir et al., 2010).

2.6. Image analysis

Intensity analysis of specific regions of interests (ROIs: background, soma, diffuse, clustered) was done in ImageJ 1.43u which allowed the export of raw data to MatlabR2008a Software. Image correction was done in ImageJ software using the plugin StackReg macro (Thévenaz et al., 1998) which corrects for drift in (x,y). Inverted average intensity projection was done in ImageJ by using the Z-stack application from frame 1–10 (0–3 min) and frame 30–60 (10–20 min). Analysis of SEP-imaging raw data was done using Matlab Software through a custom designed code. Background was subtracted from each frame, fluorescence intensity was normalised to the baseline (average value of $t = 0\text{--}3.33$ min) and averaged for each experimental group. The fluorescence intensity values for each specific ROI were analysed in individual loops which allowed separate analysis. Furthermore, the standard error of mean (SEM) was calculated for each time-point and the mean normalised values including error bars were plotted against time. Ca^{2+} imaging was analysed using ImageJ. Fluorescence intensity in the soma was extracted from one ROI per cell. Baseline for 60 min Ca^{2+} -imaging was the average of the first 10 frames ($t = 0\text{--}50$ s), which corresponds to control conditions.

2.7. Statistical analysis

All experiments were performed on neurons from at least three individual preparations. The software GraphPad Prism was used for statistical tests and to generate bar charts. Data sets were tested to determine if they were normally distributed (KS normality test) before undertaking further statistical analysis. For low Mg^{2+} only and low Mg^{2+} NMDA treatments, *p* values were determined using a Student's *t* test (two-tailed). Repeat measures ANOVA (for normally distributed data) or Friedman test was used to analyse significance of low Mg^{2+} induced effects during Ca^{2+} -imaging, low Mg^{2+} /dAPV and low Mg^{2+} /CAIP experiments since there were more than two experimental groups. Appropriate post-hoc tests such as Tukey's for normally distributed data or Dunn's multiple comparison for non-normally distributed data were used. Values are given as mean \pm SEM. Error bars represent SEM.

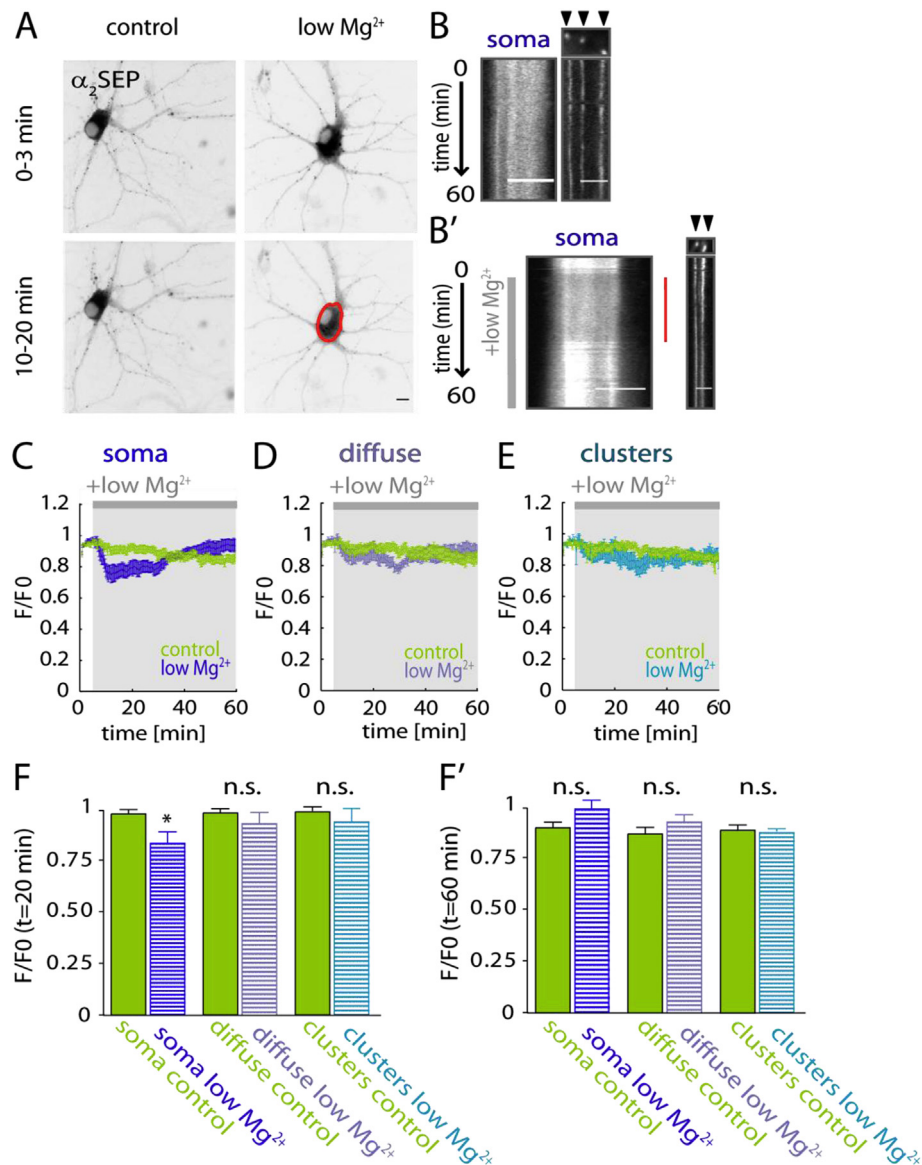


Fig. 1. Somatic surface α_{2SEP} -GABA_ARs decrease upon low Mg^{2+} treatment. (A) Representative average intensity projection of α_{2SEP} expression in control and low Mg^{2+} treated neurons over time (0–3 min and 10–20 min). Scale bar, 10 μm . (B) Kymograph (a line scan vertically projected over time) showing somatic (left; scale bar, 5 μm) and clustered (right; arrow heads indicate clusters; scale bar, 2 μm) α_{2SEP} fluorescence intensity over the movie in control (aCSF) conditions and in the presence of low Mg^{2+} (grey bar). Red bar on the right indicates a decrease in somatic fluorescence intensity upon low Mg^{2+} treatment. (C) Average fluorescence intensity of somatic α_{2SEP} GABA_AR F/F_0 : control (green, $n = 9$ cells) and low Mg^{2+} (blue, $n = 7$). (D) Time course of diffuse α_{2SEP} GABA_AR F/F_0 : control (green, $n = 9$ cells); low Mg^{2+} (purple, $n = 7$). (E) Time course of α_{2SEP} GABA_AR clusters F/F_0 : control (green, $n = 9$ cells) and low Mg^{2+} (light blue, $n = 7$). (F) Bar graph of ROI's F/F_0 : soma (left), diffuse (middle) clusters (right). Significant loss of fluorescence in the soma compared to control at 20 min following low Mg^{2+} treatment ($p = 0.02$). Diffuse fluorescence is not altered at 20 min ($p = 0.36$) after low Mg^{2+} treatment. Fluorescence intensity of α_{2SEP} GABA_AR clusters is unaltered following low Mg^{2+} treatment ($t = 20$ min; $p = 0.28$) compared to control. (F') At 60 min after low Mg^{2+} treatment somatic ($p = 0.09$), diffuse ($p = 0.85$) and clustered ($p = 0.42$) fluorescence intensity are not significantly altered. * $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Surface stability of somatic GABA_ARs in hippocampal neurons is altered during Low Mg^{2+} treatment

To examine the influence of SE on GABA_AR stability and clustering *in vitro*, we mimicked the characteristic repetitive epileptiform bursting activity of SE by removal of Mg^{2+} from the extracellular medium of cultured hippocampal rat neurons transfected with SEP-tagged GABA_AR α_2 subunit (α_{2SEP}) (Sombati and Delorenzo, 1995). Surface GABA_ARs were imaged via the SEP-tag on the α_2 subunit (Muir et al., 2010) (which allows visualisation

through high fluorescence in neutral pH, Sup. Fig.1) for 60 min. α_{2SEP} fluorescence was analysed in 3 distinct regions of interests (ROIs): soma, diffuse (extrasynaptic compartment in dendrites) and clusters. At $t = 20$ min, somatic fluorescence of α_{2SEP} -containing GABA_ARs was significantly decreased (control F/F_0 : 0.997 ± 0.02 , low Mg^{2+} F/F_0 : 0.85 ± 0.05 ; $p = 0.02$) indicating that internalisation of GABA_ARs at the somatic level increases during low Mg^{2+} treatment (Fig. 1C,F). This could account for a decrease in hippocampal GABAergic inhibition during epileptiform activity. However, α_{2SEP} -fluorescence intensity at the soma was not found to be significantly changed at $t = 60$ min (control F/F_0 : 0.92 ± 0.03 , low Mg^{2+} F/F_0 : 1.01 ± 0.04 ; $p = 0.09$) suggesting a biphasic regulation of surface

GABA_ARs during low Mg²⁺ treatment (Fig. 1C,F). Interestingly, α_2 SEP-GABA_AR clusters ($t = 20$ min; control F/F_0 : 1.02 ± 0.02 low Mg²⁺ F/F_0 : 0.96 ± 0.07 ; $p = 0.36$) and diffuse ($t = 20$ min; control F/F_0 : 1.01 ± 0.02 , low Mg²⁺ F/F_0 : 0.95 ± 0.05 ; $p = 0.28$) fluorescence intensity in the neuronal dendrites during low Mg²⁺ treatment showed only a minor, non-significant decrease. Our data thus indicates compartmental specificity of low Mg²⁺ induced decrease of GABA_ARs from the surface (Fig. 1D,E), with GABA_ARs primarily endocytosed from the cell soma surface.

3.2. Activity of NMDA receptors induces the down-regulation of somatic GABA_A receptors from the surface during Low Mg²⁺ treatment

Low extracellular Mg²⁺ induces epileptiform activity which is abolished by application of the NMDAR antagonist dAPV (Coan and Collingridge, 1985, 1987; Tancredi et al., 1990; Albowitz et al., 1997; Westerhoff et al., 1995; Gulyás-Kovács et al., 2002; Mangan and Kapur, 2004). Therefore, we tested whether inhibition of NMDAR activity during low Mg²⁺ treatment blocks the somatic down-regulation of GABA_ARs (Fig. 2). Low Mg²⁺ alone induced a significant decrease of α_2 SEP-GABA_AR fluorescence intensity at $t = 20$ min whereas this loss was inhibited by the co-application of dAPV ($t = 20$ min; control F/F_0 : 0.97 ± 0.05 , low Mg²⁺ F/F_0 : 0.65 ± 0.03 ($p < 0.001$), low Mg²⁺/dAPV F/F_0 : 0.96 ± 0.07 ($p > 0.05$); one-way ANOVA test, Tukey's multiple comparison post test), confirming that the down regulation of surface GABA_ARs was dependent on the activation of NMDARs (Fig. 2C,C').

3.3. Epileptiform activity evokes intracellular Ca²⁺ changes that correspond to the temporal dynamics of somatic surface GABA_AR decrease

To further explore the mechanisms of NMDAR-driven decrease in surface GABA_ARs during low Mg²⁺ treatment, we applied the fluorescent Ca²⁺ indicator fluo4 in low Mg²⁺ treated hippocampal neurons. This allowed us to investigate intracellular Ca²⁺ transients evoked by low Mg²⁺ treatment. Hippocampal neurons perfused with control aCSF exhibit small Ca²⁺ transients reflecting spontaneous activity, whereas low Mg²⁺ perfusion significantly altered intracellular Ca²⁺ throughout the timeline of 60 min (Fig. 3B,B'). Fluo4 imaging reported intracellular Ca²⁺ increases rapidly upon early perfusion with low Mg²⁺ (10–20 min F/F_0 : 375.8 ± 28.4 ; $p < 0.001$, Friedman test and Dunn's multiple comparison post test) and at $t = 60$ min (F/F_0 : 215.2 ± 28.8 ; $p < 0.05$, Friedman test and Dunn's multiple comparison post test) in comparison to baseline ($t = 100$ – 150 s; F/F_0 : 104.5 ± 4.4), (Fig. 3C). This indicates that low Mg²⁺ treatment induces an intracellular Ca²⁺ rise, which is likely to be caused by activation of NMDARs. Interestingly, intracellular Ca²⁺ concentration drops significantly during the timeline (10–20 min, F/F_0 : 375.8 ± 28.4 ; 50–60 min, F/F_0 : 215.2 ± 28.8 ; $p < 0.05$, Friedman test and Dunn's multiple comparison post test) showing that intracellular Ca²⁺ concentration undergoes alteration on a similar timescale to that of somatic surface GABA_AR decrease (Fig. 3C).

3.4. Dispersion of clustered GABA_A receptors is induced by NMDA receptor activation

It has been reported that the dispersal of surface GABA_AR clusters in neuronal processes is regulated through Ca²⁺ influx via NMDARs (Muir et al., 2010). Therefore we tested whether further increasing the activation of NMDARs by co-application of low Mg²⁺ and the agonist NMDA would trigger dispersion of surface GABA_AR clusters in proximal dendrites. Indeed, the activation of NMDARs with low Mg²⁺ in addition to application of the agonist NMDA (low

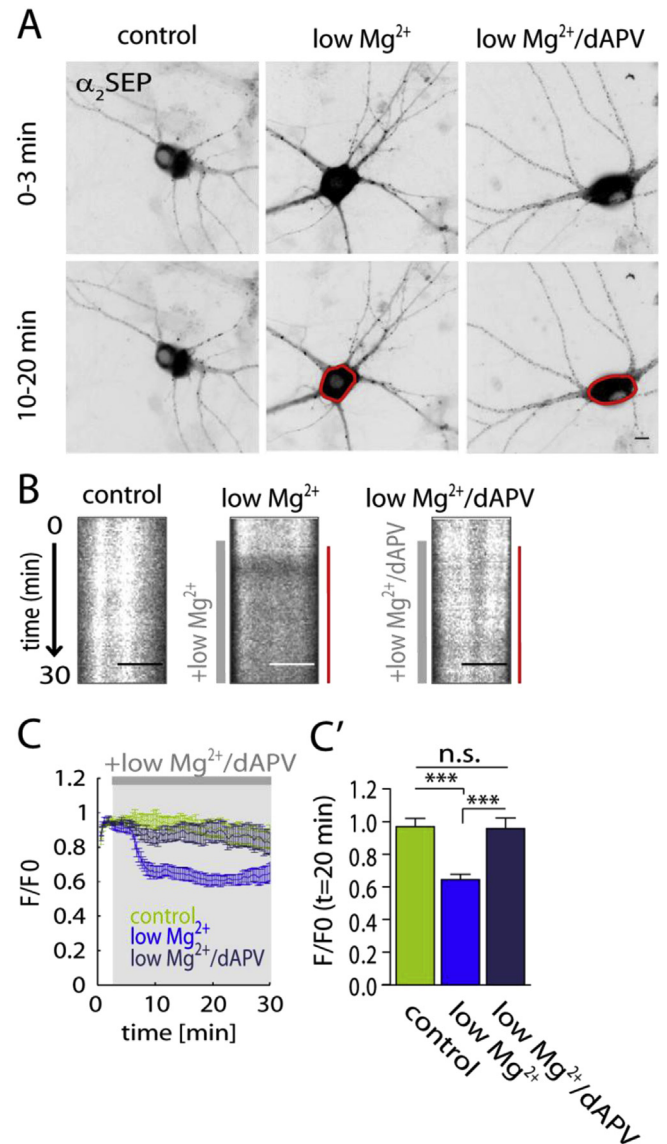


Fig. 2. NMDARs mediate low Mg²⁺ induced somatic α_2 SEP GABA_AR surface decrease. (A) Representative images of α_2 SEP GABA_AR fluorescence in control, low Mg²⁺ and low Mg²⁺ with dAPV (low Mg²⁺/dAPV) treated neurons as an average intensity projection over time (0–3 min and 10–20 min). Somatic α_2 SEP GABA_AR loss highlighted in red; scale bar, 10 μ m. (B) Kymograph showing somatic (left; scale bar, 5 μ m) fluorescence intensity over the movie (duration: 60 min) in control (aCSF) conditions and in the presence of low Mg²⁺ and low Mg²⁺/dAPV (grey bar). Red bar on the right indicates decrease in somatic fluorescence intensity upon low Mg²⁺ treatment and blocking of low Mg²⁺ induced effect by dAPV (B). (C) Time course of somatic α_2 SEP GABA_AR F/F_0 : control (green, $n = 7$ cells), low Mg²⁺ (blue, $n = 8$ cells) and low Mg²⁺/dAPV (dark blue, $n = 6$ cells). (C') Summary of somatic F/F_0 at 20 min after low Mg²⁺ treatment. Low Mg²⁺ induces a significant decrease ($p < 0.001$) in somatic fluorescence intensity, which is inhibited by application of NMDAR blocker dAPV ($p < 0.001$). *** $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Mg²⁺/NMDA) caused a loss of α_2 SEP GABA_AR fluorescence intensity in dendritic clusters (Fig. 4A,B,B') at $t = 20$ min (control F/F_0 : 1.001 ± 0.04 , low Mg²⁺/NMDA F/F_0 : 0.799 ± 0.03 ; $p < 0.01$) suggesting that surface stability of GABA_ARs corresponds with the potency of NMDAR activation (Fig. 4C,F). Interestingly, during low Mg²⁺/NMDA perfusion diffuse (control F/F_0 : 1.02 ± 0.03 , low Mg²⁺/NMDA F/F_0 : 0.95 ± 0.09 , $p = 0.58$) and total (data not shown) fluorescence intensity in neuronal processes remains unaltered at $t = 20$ min (Fig. 4D,F). This indicates dispersion of surface GABA_AR

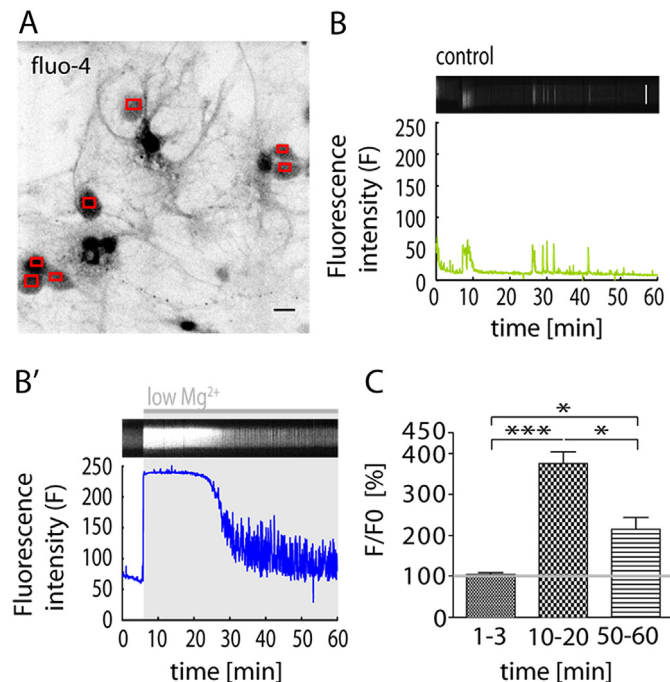


Fig. 3. Low Mg^{2+} treatment induces intracellular Ca^{2+} accumulation. (A) Representative average intensity projection of fluo-4 loaded neurons (coloured squares indicate individual cell bodies). (B) Raw fluorescence intensity of spontaneous Ca^{2+} transients in a neuron (bottom) and correlating kymograph (top; segmented line through somatic ROI) showing fluorescence changes over time under control (aCSF) conditions. (B') Raw fluorescence intensity reporting low Mg^{2+} (grey bar) induced Ca^{2+} accumulation (bottom) and kymograph (top) indicating increase in fluorescence intensity. (C) Quantification of increase in intracellular Ca^{2+} ($n = 21$ cells). Between 10 and 20 min (averaged data points from min 10 to min 20) after low Mg^{2+} induction, intracellular Ca^{2+} is significantly elevated ($p < 0.001$) compared to baseline (averaged data points from min 0 to min 3). At 50–60 min intracellular Ca^{2+} has decreased ($p < 0.001$) compared to 10–20 min and significantly increased ($p = 0.02$) compared to the baseline. * $p < 0.05$, *** $p < 0.001$. Scalebar, 10 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

upon low Mg^{2+} /NMDA. Somatic GABA_AR fluorescence intensity is significantly decreased at $t = 10$ min after low Mg^{2+} /NMDA (control F/F_0 : 1.00 ± 0.02 , low Mg^{2+} /NMDA F/F_0 : 0.77 ± 0.059 , $p < 0.01$) treatment, however it is not significantly altered at $t = 20$ min (control F/F_0 : 0.98 ± 0.03 , low Mg^{2+} /NMDA F/F_0 : 0.85 ± 0.11 , $p = 0.34$) (Fig. 4 E). Although the decrease in somatic surface GABA_AR fluorescence intensity during low Mg^{2+} /NMDA also occurs rapidly after treatment and is of similar size compared to low Mg^{2+} only treatment, the biphasic recovery phase is shorter in the low Mg^{2+} /NMDA treatment.

3.5. Calcineurin mediates the decrease of GABA_A receptors from the surface during Low Mg^{2+} induced bursting activity

We next investigated the signalling mechanisms involved in NMDAR mediated GABA_AR surface decrease during epileptiform bursting activity. Calcineurin is implicated in activity-dependent regulation of GABAergic inhibition and hence could play an important role in Ca^{2+} mediated signalling, we therefore analysed its role in GABA_AR stability during low Mg^{2+} bursting (Lu et al., 2000; Wang et al., 2003; Chen and Wong, 1995; Muir et al., 2010). Cells undergoing epileptiform activity showed a decrease in somatic GABA_AR fluorescence intensity compared to control. We found that treating cells with a calcineurin autoinhibitory peptide did not significantly affect somatic GABA_AR intensity (control F/F_0 : 1.07 ± 0.10 , control/CAIP F/F_0 : 0.95 ± 0.03 ; $p > 0.05$, one-way

ANOVA test and Tukey's multiple comparison post test) showing that calcineurin had no effect at $t = 20$ min in control conditions (Fig. 5C,C'). However, blocking calcineurin activity, inhibited the low Mg^{2+} -induced decrease of surface GABA_AR at the soma at $t = 20$ min significantly (low Mg^{2+} F/F_0 : 0.75 ± 0.06 , low Mg^{2+} /CAIP F/F_0 : 1.043 ± 0.06 ; $p < 0.05$, one-way ANOVA test and Tukey's multiple comparison post test) (Fig. 5C,C'). These results suggest that calcineurin activation upon Ca^{2+} influx through NMDARs is directly involved in the decrease of surface GABA_AR triggered by epileptiform bursting activity.

4. Discussion

GABA_ARs are a target for a variety of drugs including benzodiazepines, which are of high clinical relevance for first-line treatment of SE. Therefore it is likely that the modulation of surface stability of GABA_AR induces or supports benzodiazepine pharmacoresistance in patients with SE. To identify potential mechanisms facilitating GABA_AR internalisation, we performed live-imaging on SEP-tagged GABA_AR. The key finding of this study shows epileptiform activity induces activation of calcineurin leading to a decrease in the number of surface GABA_AR in the soma. This activity-dependent alteration of inhibitory strength is mediated by activation of NMDARs and is paralleled by an increase in intracellular Ca^{2+} which in turn is likely to activate calcineurin. These data identify a signalling mechanism underlying surface GABA_AR decrease during SE and supports studies that report NMDAR activation regulates GABAergic inhibition (Muir et al., 2010; Bannai et al., 2009; Stelzer et al., 1987).

It is known that internalisation of GABA_AR containing α , γ_2 or $\beta_{2/3}$ subunits is increased during epileptiform activity *in vitro* using low Mg^{2+} or high KCl media (Blair et al., 2004; Goodkin et al., 2008, 2005). Furthermore, it has been demonstrated that GABA_ARs are internalised in chemoconvulsant models of SE *in vivo* (Naylor et al., 2005; Nishimura et al., 2005). Our data supports the hypothesis that GABA_AR internalisation occurs during prolonged seizures, by demonstrating that GABA_AR which contain the α_2 subunits are decreased during low Mg^{2+} treatment. In the majority of cells this decrease in surface GABA_AR was biphasic possibly indicating an adaptational switch in inhibitory strength. Interestingly, we observe that this effect occurs preferentially in the soma but less in dendrites. Compartmental internalisation of GABA_AR during SE has not been reported and the mechanisms underlying this differential regulation are unknown, but there are a number of possibilities. Firstly, it remains to be investigated to what extent intracellular Ca^{2+} buffering systems such as endoplasmic reticulum or mitochondria are contributing to surface stability of GABA_AR in neurons undergoing epileptiform activity. Expression of signalling proteins in specific subcellular compartments could explain this effect. Secondly, since this study makes use of experiments based on overexpression of GABA_AR, this could account for increased inhibition which could suppress a decrease of GABA_AR in dendrites specifically. Omitting Mg^{2+} in the extracellular medium of cultured hippocampal neurons triggers a sequence of events leading to neuronal death (Kovac et al., 2012; Yoon et al., 2010). To significantly reduce neuronal cell death, mitochondrial substrate sodium pyruvate was added in our experiments. Although we control for this substitution, it would be interesting to test whether mitochondrial ATP production affects GABA_AR trafficking during prolonged seizures. Thirdly, correlating the amount of intracellular ATP to the trafficking of GABA_AR could contribute to the understanding of compartmentalised trafficking of GABA_AR during low Mg^{2+} treatment.

A comprehensive body of literature describes that low Mg^{2+} induced epileptiform activity is dependent on increased NMDAR

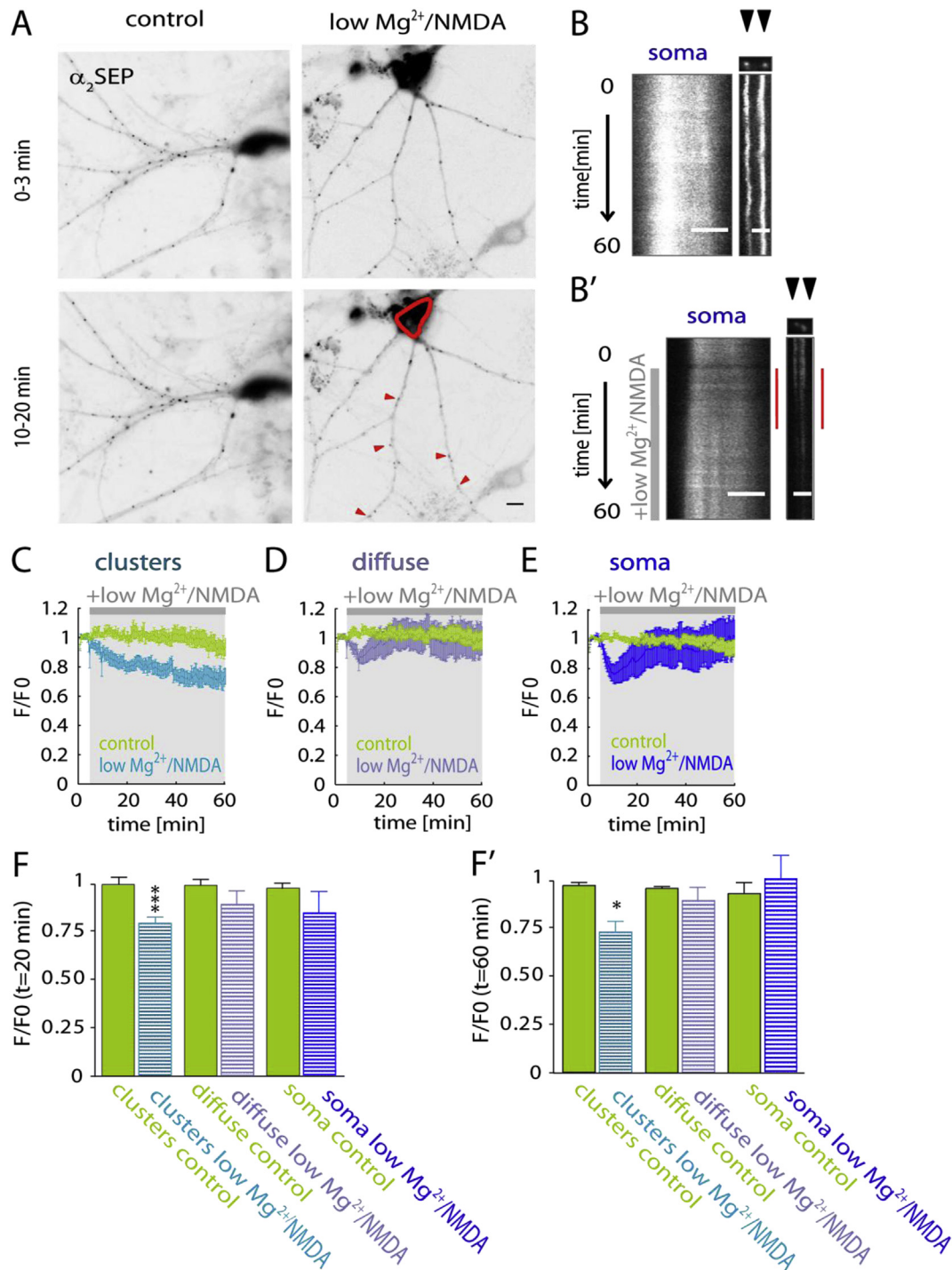


Fig. 4. Clustered $\alpha_{2\text{SEP}}$ GABA_ARs decrease upon low Mg^{2+} /NMDA treatment. (A) Representative average intensity projection of $\alpha_{2\text{SEP}}$ GABA_AR fluorescence in control and low Mg^{2+} /NMDA treated neurons over time (0–3 min and 10–20 min). Somatic $\alpha_{2\text{SEP}}$ GABA_AR fluorescence highlighted in red; scale bar, 10 μm . (B) Kymograph showing somatic (left; scale bar, 5 μm) and clustered (right; scale bar, 2 μm) $\alpha_{2\text{SEP}}$ GABA_AR fluorescence intensity over the movie in control (aCSF) conditions and in the presence of low Mg^{2+} /NMDA (grey bar). Red bar on the right indicates decrease in somatic $\alpha_{2\text{SEP}}$ GABA_AR fluorescence intensity upon low Mg^{2+} /NMDA treatment. (C) Average fluorescence intensity time course of clustered $\alpha_{2\text{SEP}}$ GABA_AR F/F_0 : control (green, $n = 7$ cells) and low Mg^{2+} /NMDA (light blue, $n = 9$). (D) Time course of diffuse $\alpha_{2\text{SEP}}$ GABA_AR F/F_0 : control (green, $n = 7$ cells); low Mg^{2+} , (purple, $n = 9$). (E) Time course of somatic $\alpha_{2\text{SEP}}$ GABA_AR F/F_0 : control (green, $n = 7$ cells) and low Mg^{2+} (blue, $n = 9$). (F) Bar graph of ROI's F/F_0 : clusters (left), diffuse (middle), soma (right). Significant loss of fluorescence in the clusters compared to control at 20 min following after low Mg^{2+} treatment ($p = 0.0008$). Diffuse fluorescence is not altered upon low Mg^{2+} treatment at 20 min ($p = 0.36$) after low Mg^{2+} treatment. Diffuse fluorescence is still significantly reduced ($p < 0.001$), diffuse ($p = 0.99$) and somatic ($p = 0.63$) fluorescence are not significantly altered. (F') At 60 after low Mg^{2+} treatment clustered fluorescence intensity is still significantly reduced ($p < 0.001$), diffuse ($p = 0.99$) and somatic ($p = 0.63$) fluorescence are not significantly altered. * $p < 0.05$, *** $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

activity (Coan and Collingridge, 1985, 1987; Tancredi et al., 1990; Albowitz et al., 1997; Westerhoff et al., 1995; Gulyás-Kovács et al., 2002; Mangan and Kapur, 2004), therefore we tested whether the somatic surface GABA_AR decrease was mediated by NMDAR

activity. Our experiments confirm this hypothesis by reporting that the decrease in surface GABA_AR was blocked by application of NMDAR blocker dAPV. To our knowledge this is the first study showing a direct regulation of GABA_ARs by NMDAR activation

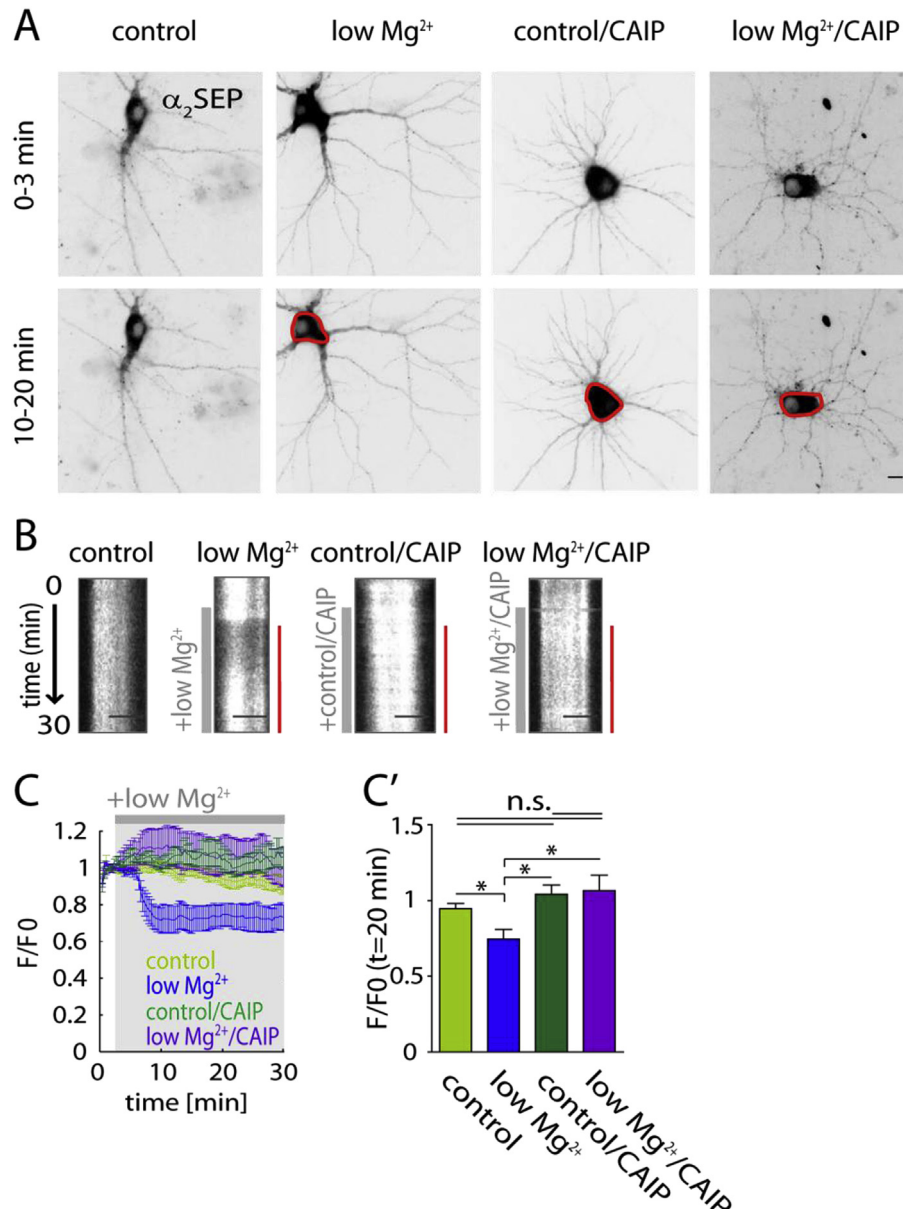


Fig. 5. Calcineurin mediates the decrease of somatic surface GABA_ARs during low Mg^{2+} treatment. (A) Representative images of α_2SEP GABA_AR fluorescence in control, control with CAIP (control/CAIP), low Mg^{2+} and low Mg^{2+} with CAIP (low Mg^{2+} /CAIP) treated neurons as an average intensity projection over time (0–3 min and 10–20 min). Scale bar, 10 μm . (B) Kymographs showing somatic (scale bar: 5 μm) fluorescence intensity over the movie (duration: 30 min) in control conditions, control/CAIP and in the presence of low Mg^{2+} and low Mg^{2+} /CAIP (grey bar). Red bar on the right indicates decrease in somatic fluorescence intensity upon low Mg^{2+} treatment. (C) Average fluorescence intensity of α_2SEP GABA_AR F/F₀: control (light green, $n = 6$ cells); low Mg^{2+} (dark blue, $n = 7$ cells); control/CAIP (dark green, $n = 6$); low Mg^{2+} /CAIP (purple, $n = 6$). (C') Bar graph showing quantification of α_2SEP GABA_AR F/F₀ at $t = 20$ min. Somatic fluorescence intensity of low Mg^{2+} treated cells is significantly decreased compared to control at $t = 20$ min (dark blue bar, $p < 0.05$). Treatment of low Mg^{2+} perfused cells with a calcineurin autoinhibitory peptide prevents the change in fluorescence intensity (dark green bar, $p < 0.05$). Treatment with calcineurin autoinhibitory peptide alone does not significantly alter the fluorescence intensity of GABA_AR α_2SEP (magenta bar, $p > 0.05$). * $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

during low Mg^{2+} induced epileptiform activity. However, whether the same process underlies the regulation of GABA_ARs during epileptiform activity induced by different approaches (Goodkin et al., 2008; Laurén et al., 2005) remains to be determined. The observation that NMDAR activation and rises in internal calcium are common to all models of SE (Raza et al., 2004; Rice and DeLorenzo, 1998; Mazarati and Wasterlain, 1999) would suggest that this is a universal mechanism.

Interestingly, the direct activation of NMDARs with low Mg^{2+} and NMDA induces a potent change in surface stability of GABA_AR clusters. The mechanisms underlying this modulation need further

investigation, however dephosphorylation of GABA_AR $\gamma 2$ subunits could play a role (Muir et al., 2010). Our data suggest that potency of NMDAR activation correlates with the extent of GABA_AR modification, and may explain the synergistic effect of benzodiazepines and NMDAR antagonists in the treatment of SE (Rice and DeLorenzo, 1999; Martin and Kapur, 2008).

Ca^{2+} influx from the extracellular environment through NMDARs can alter the stability of inhibitory neurotransmitter receptors (Bannai et al., 2009; Muir et al., 2010). Bannai et al. showed that diffusion dynamics of GABA_ARs too are tuned by Ca^{2+} entry from the extracellular space. Indeed, here we report an increase in

intracellular Ca^{2+} concentration during low Mg^{2+} treatment. Moreover, Ca^{2+} levels correlate with the temporal dynamics of the low Mg^{2+} induced effect on GABA_ARs. This indicates that fast Ca^{2+} -signalling could indirectly be altering GABA_ARs surface stability. Although technically challenging, it would be of major interest to simultaneously dual record Ca^{2+} dynamics and GABA_ARs surface stability to better address the relationship of low Mg^{2+} induced spiking activity and GABA_AR trafficking on a single cell level.

Surface stability of GABA_ARs is regulated by multiple processes which are facilitated by direct or indirect interaction with trafficking proteins (Marsden et al., 2007; Jacob et al., 2008). Our experiments further emphasise the relationship of indirect signalling via intracellular Ca^{2+} sensing proteins to stabilise neurotransmitter receptors. Downstream effects of Ca^{2+} sensing proteins such as calmodulin orchestrate a number of target proteins and can trigger selective effects on surface GABA_AR stability. It is known that increased phosphorylation of GABA_ARs contributes to surface stability (Saliba et al., 2012). Terunuma et al. demonstrated deficits in GABA_AR phosphorylation during SE mediated by protein kinase C (Terunuma et al., 2008). Oppositely, dephosphorylation induces declustering and increases the diffusion dynamics of GABA_ARs (Muir et al., 2010). Calcineurin has been shown to interact with GABA_ARs via the γ_2 subunit and it modulates neuronal inhibition. Interestingly, basal and maximal activity of calcineurin is increased (Kurz et al., 2001) and subcellular distribution is altered (Kurz et al., 2003) in SE *in vivo*. However, it has been poorly investigated whether calcineurin mediates the decrease in surface GABA_ARs (Wang et al., 2009). We identify calcineurin as a mediator of the decrease in surface GABA_AR and therefore, provide a mechanism of inhibitory modulation during SE, and a potential target for therapy. Moreover, this study is the first to demonstrate a decrease in surface GABA_ARs by live-imaging in hippocampal neurons during SE. The identification of the underlying trafficking mechanism could account for resistance to benzodiazepines and could have additive effects on duration or frequency of seizure activity. Further research is needed to develop more effective therapeutic strategies against SE to which this study will contribute.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.neuropharm.2014.09.014>.

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